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High diagnostic yield with algorithmic molecular approach on hereditary neuropathies

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SUMMARY

OBJECTIVE: Charcot-Marie-Tooth disease covers a group of inherited peripheral neuropathies. The aim of this study was to investigate the effect of targeted next-generation sequencing panels on the molecular diagnosis of Charcot-Marie-Tooth disease and its subtypes in routine clinical practice, and also to show the limitations and importance of next-generation sequencing in the diagnosis of Charcot-Marie-Tooth diseases.

METHODS: This is a retrospective study. Three different molecular methods (multiplex ligation probe amplification, next-generation sequencing, and whole-exome sequencing) were used to detect the mutations related to Charcot-Marie-Tooth disease.

RESULTS: In total, 64 patients (33 males and 31 females) with suspected Charcot-Marie-Tooth disease were analyzed for molecular etiology. In all, 25 (39%) patients were diagnosed by multiplex ligation probe amplification. With an extra 11 patients with normal PMP22 multiplex ligation probe amplification results that were consulted to our laboratory for further genetic analysis, a total of 50 patients underwent next-generation sequencing for targeted gene panels associated with Charcot-Marie-Tooth disease. Notably, 18 (36%) patients had pathogenic/likely pathogenic variants. Whole-exome sequencing was performed on five patients with normal next-generation sequencing results; the diagnostic yield by whole-exome sequencing was 80% and it was higher in the childhood group.

CONCLUSION: The molecular etiology in Charcot-Marie-Tooth disease patients can be determined according to pre-test evaluation, deciding the inheritance type with pedigree analysis, the clinical phenotype, and an algorithm for the genetic analysis. The presence of patients without a molecular diagnosis in all the literature suggests that there are new genes or mechanisms waiting to be discovered in the etiology of Charcot-Marie-Tooth disease.

KEYWORDS: Charcot-Marie-Tooth disease. DNA copy number variations. High-throughput nucleotide sequencing. Exome sequencing.

INTRODUCTION

Charcot-Marie-Tooth disease (CMT) covers a group of inherited peripheral neuropathies. It is also called hereditary motor sensory neuropathy. These neuropathies have heterogeneous clinics in terms of their phenotypic features, inheritance modes, and gene mutations in the etiology[1]. The prevalence is 9.7–82/100.000[2].

The mode of inheritance and genetic cause are important in the classification of CMT[3]. The phenotype of classical CMT contains typically distal weakness (a length-dependent motor sensory neuropathy), a high incidence of foot deformities, and sensory loss. This phenotype can occur in the first/second decade of life in most patients. There is a slow progression of these symptoms and worsening by the time[2]. Nerve conduction studies had a huge help in confirming and classifying CMTs by categorizing patients broadly into demyelinating and axonal or mixt type forms. The key parameters measured by electromyography (EMG) are distal latencies, amplitudes, and velocities of motor and sensory nerves, but the main finding is the median nerve conduction velocity, and 38 m/s is the commonly used cutoff value for differentiating demyelinating from axonal types of CMTs[4].

Genetic heterogeneity of CMT has been revealed by the common use of next-generation sequencing (NGS). Until now, more than 100 genes have been described as having causative mutations for CMT[5]. Especially, four genes are responsible for nearly 80% of genetically inherited CMTs: *PMP22*, *GJB1*, *MFN2*, and *MPZ*[6]. The most common type of CMT is CMT1A, which accounts for nearly 60% of genetically inherited CMT cases. A 1.4 Mb duplication in the short arm of chromosome 17 causes CMT1A, and this region encloses nine genes, including *PMP22* gene[7]. Another inherited neuropathy with pressure palsies has been caused by a deletion in the same gene. This points out the importance of *PMP22* gene and its protein expression level for peripheral nerve

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function. *GJB1*, *MFN2*, and *MPZ* are responsible for CMTX1, CMT2A, and CMT1B, respectively[3]. CMT2A can present in early childhood or infancy period, and it is caused by *MFN2* gene mutations with a more severe phenotype[8].

Recently, NGS has become more cost-effective, suitable, and wide for many genetically inherited diseases, including CMT. Targeted NGS panels include some causative genes related to the diseases[9,10]. This study aimed to describe the effect of targeted NGS panels on the molecular diagnosis of CMT and its subtypes in routine clinical practice, and also to show the limitations and importance of NGS at the diagnosis of CMTs.

METHODS

We reviewed the data of 64 patients who applied for hereditary peripheral neuropathy at the Ankara City Hospital Genetic Diseases Evaluation Center from February 2019 to December 2020. The patients were examined by their pediatric/adult neurologists and were referred to our genetic laboratory for a diagnostic genetic test. Patients who had acquired neuropathy were excluded. Permission for the study was obtained from the Ankara Yıldırım Beyazıt University Ethics Committee (17.02.2021/02). The study followed the guidelines and principles of the Declaration of Helsinki. All patients and formal guardians of the patients under 18 years had signed the written informed consent for the usage of their clinical data and genetic analysis. Genomic DNA was extracted from peripheral blood using QIAcube® automatic DNA isolation system (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. MRC Holland (Amsterdam, Holland) P033 CMT1 kit was used for multiple ligation-dependent probe amplification (MLPA) method according to the manufacturer's instructions. MLPA using genomic DNA extracted from whole blood was performed to detect the deletion/duplication mutations of *PMP22* gene. Qiagen CMT panel CDHS-17346Z-1897 kit (Qiagen, Hilden, Germany) was used for NGS to detect the single nucleotide variants for the targeted genes (Table 1). The target enrichment process was followed by

Table 1. Charcot-Marie-Tooth disease-related genes (44) included in targeted NGS panel, their corresponding transcript numbers, and heredity types.

Gene name	Transcript ID	Inheritance	Gene name	Transcript ID	Inheritance
AARS	NM_001605.2	AD	KIF5A	NM_004984.2	AD
ARHGEF10	NM_014629.2	AD	LITAF	NM_001136473.1	AD
BSCL2	NM_032667.6	AD,AR	LMNA	NM_170707.3	AD,AR
COX6A1	NM_004373.3	AR	MARS	NM_004990.3	AD
DHTKD1	NM_018706.6	AD	MED25	NM_030973.3	AR
DNMT1	NM_001379.2	AD	MFN2	NM_014874.3	AD,AR
DNM2	NM_001005360.2	AD	MPZ	NM_000530.6	AD
DYNC1H1	NM_001376.4	AD	MTMR2	NM_016156.5	AR
EGR2	NM_000399.3	AD	NDRG1	NM_006096.3	AR
FAM134B	NM_001034850.3	AR	NEFL	NM_006158.4	AD,AR
FIG4	NM_014845.5	AR	PLEKHG5	NM_198681.3	AR
FGD4	NM_139241.2	AR	PMP22	NM_153321.2	AD
GAN	NM_022041.3	AR	PRPS1	NM_002764.3	XLR
GARS1	NM_002047.2	AD	PRX	NM_181882.2	AR,AD
GDAP1	NM_018972.2	AR,AD	RAB7A	NM_004637.5	AD
GJB1	NM_000166.5	XLD	REEP1	NM_022912.2	AD
HSPB1	NM_001540.3	AD	SBF1	NM_002972.2	AR
HSPB8	NM_014365.2	AD	SBF2	NM_030962.3	AR
IGHMBP2	NM_002180.2	AR	SH3TC2	NM_024577.3	AR,AD
IKBKAP	NM_003640.3	AR	TRPV4	NM_021625.4	AD
INF2	NM_022489.3	AD	VCP	NM_007126.3	AD
KIF1B	NM_015074.3	AD	YARS1	NM_003680.4	AD

AD: autosomal dominant; AR: autosomal recessive; XLD: X-linked dominant; XLR: X-linked recessive.

sequencing of the libraries on Illumina MiSeq system (Illumina Inc., San Diego, CA, USA). Whole-exome sequencing (WES) was performed on five patients who had negative duplication/deletion analysis and targeted NGS panel.

Data analysis and variant interpretation

Data analysis was carried out by QIAGEN Clinical Insight (QCITM) software (QIAGEN, Hilden, Germany). Pathogenic, likely pathogenic, and uncertain significant variants were confirmed by Sanger sequencing. The exons of all targeted genes were sequenced at a read depth of 30× or greater. The 2015 American College of Medical Genetics Standards and Genomics (ACMG) was used for the interpretation of sequence variants[11].

RESULTS

In total, 64 patients (33 males/31 females) with suspected CMT were analyzed for molecular etiology. The range of the patient ages was between 3 and 74 years; 33 male cases had a mean age of 26.4 years, and 31 female cases had a mean age of 25.3 years.

First, MLPA was performed for deletion/duplication analysis for all of the patients. In all, 25 (39%) patients were diagnosed by MLPA. *PMP22* duplication was detected in 14 patients, and *PMP22* deletion was detected in 11 patients. An extra 11 patients with normal *PMP22* MLPA results who were consulted to our laboratory for further genetic analysis were also included in the study. Eventually, 50 patients with normal *PMP22* MLPA results underwent NGS for targeted gene panels associated with CMT.

Notably, 18 (36%) patients including 10 males and 8 females had pathogenic/likely pathogenic variants at *INF2*, *EGR2*, *HSPB1*, *GJB1*, *GNB4*, *LITAF*, *GDAP1*, *MFN2*, *IGHMBP2*, *SH3TC2*, *GAN*, *SBF1*, *MRM2*, and *PLA2G6* genes. Nine (50%) patients were under 18 years old. Family history was positive for six patients and consanguinity marriage for seven parents. Nine patients had homozygote pathogenic/likely pathogenic variants for genes (*IGHMBP2*, *SH3TC2[2]*, *GDAP1[3]*, *GAN*[2], and *SBF1*) that have autosomal recessive manner. Eight patients had heterozygote pathogenic/likely pathogenic variants for genes (*INF2[2]*, *EGR2*, *HSPB1*, *GNB4*, *LITAF*, *GDAP1*, and *MFN2*) that have autosomal dominant and X-linked manner (*GJB*1), respectively. In only one patient, a heterozygote pathogenic variant had been detected for an autosomal recessive inherited gene (*GAN*) (Table 2).

A total of 17 (13%) variants on 13 patients were assessed as variants of unknown significance in our study (Table 2). Five (38%) patients were under 18 years old. Pathogenic, likely pathogenic, and variant of uncertain significant variants (VUS) were confirmed by bidirectional Sanger sequencing. Over 99% of the coding exons of all genes in the panel were sequenced to a read depth of 30× or greater in almost all cases. According to these results, the molecular diagnosis rate was 39%.

WES was performed as further examination in 5 patients (3 of them under 18 years old) whose panel results were found to be normal. Pathogenic and likely pathogenic variants had been detected at four different genes in these patients (Table 3). As a result, among 22 pediatric patients, 17 were diagnosed by NGS and WES, and 19 out of 28 adult patients were also diagnosed. So, the diagnosis rates for the pediatric age group and adult groups were 55 and 39%, respectively, excluding VUS.

DISCUSSION

CMT diseases are a very wide spectrum of hereditary neuropathies that are caused by a large number of different genes[12]. There is a genetic heterogeneity in the inheritance of the genes responsible for CMTs. The molecular pathways of these genes related to CMTs are quite complex; thus, the diagnosis is also complicated[12]. At this point, a new approach is needed for the correct diagnosis[6].

PMP22 duplication/deletion test is the first diagnostic method for CMT1. In our study, MLPA was the first method used for the investigation of duplication/deletion analysis for *PMP22* gene. The diagnostic yield for MLPA was 39%, which was nearly compatible with the literature[7].

If the MLPA test is negative or there is another type of CMT, a targeted NGS gene panel should be performed[13]. With these targeted gene panels listing all known disease-causing genes, a large group of genes can be sequenced and analyzed to show the different variants (pathogenic, likely pathogenic, or variants of unknown significance), and this method can be accepted as the most effective genetic testing in CMT. The diagnosis rate is 18–31% for CMT gene panels, related to the sequencing quality and the included genes[14]. Vaeth et al. reported that 6.7% pathogenic/likely pathogenic variants were detected with targeted NGS panel in CMT patients[15]. The higher depth of coverage is an important factor for the higher accuracy of the test[14]. The diagnostic yield for NGS in our study was 36%. We think that the reason why this rate is slightly higher than that reported in the literature is that the right patients were chosen based on their clinical findings, EMG results, and family history. Notably, 18 patients who had undergone to NGS had pathogenic/likely pathogenic variants mostly at autosomal recessive inherited genes (*IGHMBP2*, *SH3TC2*, *GDAP1*, *GAN*, *SBF1*, *EGR2*, *MFN2*) and one at X-linked inherited gene (*GJB1*). The rest of the patients had pathogenic/likely pathogenic variants at autosomal dominant inherited genes (*INF2*, *HSPB1*,

Table 2. Cases with pathogenic/likely pathogenic variants and variant of uncertain significant variants.

Gender	Age	Result	ACMG 2015 criteria	Phenotype (OMIM)
M	14	*INF2* (NM_022489.3):c.218G>A(p.Gly73Asp) Heterozygote	Likely pathogenic (novel)	CMTDIE (614455)
M	44	*EGR2* (NM_000399.4):c.1142G>T(p.Arg381Leu) Heterozygote	Likely pathogenic (novel)	CMT1D (607678)
F	36	*INF2* (NM_022489.3):c.271C>G(p.Arg91Gly) Heterozygote	Likely pathogenic	CMTDIE (614455)
M	74	*HSPB1* (NM_001540.5):c.562C>T(p.R188W) Heterozygote	Likely pathogenic	CMT2F (606595)
F	40	*GJB1* (NM_000166.5):c.581T>C(p.M194T) Heterozygote	Likely pathogenic	CMTX (1302800)
M	28	*GNB4 (NM_021629.4):c.266A>C(p.Lys89Thr)* Heterozygote	Likely pathogenic	CMTDIF (615185)
M	9	*LITAF* (NM_004862.3):c.430G>A(p.Val144Met) Heterozygote	Likely pathogenic	CMT1C (601098)
M	10	*GDAP1* (NM_018972.4): c.836A>G(p.Tyr279Cys) Heterozygote	Pathogenic	CMT2K (607831)
F	3	*MFN2* (NM_001127660.1):c.1090C>T(p.Arg364Trp) Heterozygote	Likely pathogenic	CMT2A2A (609260)
F	5	*IGHMBP2* (NM_002180.2): c.1347G>A(p.Met449Ile) Homozygote	Pathogenic	CMT2S (616155)
M	20	*SH3TC2* (NM_024577.3):c.1896_1897delGGinsA(p.Ala633fs*12) Homozygote	Pathogenic	CMT4B2 (604563)
F	33	*SH3TC2* (NM_024577.3):c.2860C>T(p.R954*) Homozygote	Pathogenic	CMT4C (601596)
M	15	*GDAP1* (NM_018972.3):c.347T>G(p.M116R) Homozygote	Likely pathogenic	CMT2K (607831)
F	12	*GAN* (NM_022041.3):c.1369_1370dupAG(p.R458fs*32) Homozygote	Pathogenic	Giant axonal neuropathy-1
F	19	*SBF1* (NM_002972.4):c.5297G>A(p.Arg1766His) Homozygote	Likely pathogenic	CMT4B2 (604563)
M	13	*GDAP1* (NM_001040875.3):c.278G>A(p.Arg93His) Homozygote	Likely pathogenic	CMT2K (607831)
M	6	*GAN* (NM_022041.4):c.968C>A(p.S323*) Homozygote	Pathogenic	Giant axonal neuropathy-1
F	23	*GDAP1* (NM_018972.3):c.347T>G(p.M116R) Homozygote	Likely pathogenic	CMT2K (607831)
M	18	*MFN2* (NM_014874.3): c.2167G>A(p.p.Val723Ile) Heterozygote	VUS	CMT2A2A (609260)
F	32	*SBF2* (NM_030962.3):c.5014_5016delAAA(p.Lys1672del) Homozygote, *ARHGEF10* (NM_014629.3):c.2881T>G(p.Ser961Ala) Heterozygote	VUS	CMT4B2 (64563) Slowed nerve conduction velocity (608236)
F	32	*VCP* (NM_007126.5):c.34C>A(p.L12I) Heterozygote	VUS	CMT2Y (616687)
M	5	*PMP22* (NM_000304.4):c.103G>A(p.A35T) Heterozygote	VUS	CMT1A (118220) CMT1E (118300)
F	52	*TRPV4* (NM_021625.4):c.133G>A(p.G45S) Heterozygote	VUS	HMSN2C (606071)
M	17	*SBF1* (NM_002972.4):c.1637-4delG Heterozygote, *SH3TC2* (NM_024577.3):c.3293C>T(p.T1098I) Heterozygote	VUS	CMT4B2 (604563) CMT4B2 (604563)
M	16	*REEP1* (NM_022912.2):c.262T>C(p.Y88H) Heterozygote, *FIG4* (NM_014845.5):c.1246T>G(p.W416G) Heterozygote	VUS	HMN5B (614751) CMT4J (611228)
F	25	*INF2* (NM_022489.4):c.1541C>T(p.P514L) Heterozygote	VUS	CMTDIE (614455)
F	29	*PLEKHG5* (NM_020631.5):c.2362_2363TC[2] (p.Leu789fs) Heterozygote	VUS	CMTC (615376)
M	2	*DYNC1H1*:c.10619A>G(p.N3540S) Heterozygote	VUS	CMT2O (614228)
F	38	*DHTKD1* (NM_018706.7):c.857A>G(p.Asn286Ser) Heterozygote, *PLEKHG5* (NM_001042663.2):c.1778G>A(p.Arg593Gln) Heterozygote	VUS	CMT2Q (615025) CMTC (615376)
F	31	*GARS1* (NM_0013166772.1):c.1598C>T(p.T533M) Heterozygote	VUS	CMT2D (601472)
M	19	*EGR2* (NM_001136179.3):c.364_369dupCCTCCT (p.Pro172_Pro173dup) Heterozygote	VUS	CMT1D (607678)

Table 3. Molecular findings of whole-exome sequencing at chosen patients.

Gender	Age	Clinical findings	Gene (RefSeq Transcript)	Mutation nucleotide change/ Amino acid change	Zygosity and inheritance	Database info dbSNP/ HGMD/ Novel/ ClinVar	SIFT	PolyPhen	CADD score*	Frequency
Male	5	Delayed motor development, severe hypotonia, dysmorphic facial features, cleft lip	*VAMP1* (NM_199245.3)	c.202C>T/p.R68*	Homozygous/ AR	CM11716 (DM)/ rs76969393	No prediction	No prediction	36	0.00000795
Male	17	Developmental delay, neurosensory deafness, hepatosplenomegaly, liver enzyme abnormalities, palmoplantar hyperkeratosis	*MRM2* (NM_0133933)	c.638A>C/p.Gln213Pro	Homozygous/ AR	rs372352761	Tolerated	Possibly damaging	21.6	0.00000398
Male	5	Developmental delay, psychomotor regression	*PLA2G6* (NM_001199562.3)	c.1610G>A/p.Arg537Gln	Homozygous/ AR	CM063032 (DM)/ rs776713955	Damaging	Probably damaging	31	0.00000398
Male	19	Gait disturbance, distal muscle weakness, scoliosis	Normal	–	–	–	–	–	–	–
Male	47	Gait instability, distal muscle weakness	*MME* (NM_007289.4)	c.160+1G>C/ splice site	Homozygous/ AR	Novel	No prediction	No prediction	33	–

AR: autosomal recessive. *CADD is a tool for scoring the harmfulness of a variant. A score of 10 indicates that the variant is supposed to be among the top 10% of deleterious variants, a score of 20 indicates the variant is in the top 1%.

GNB4, *LITAF*, *MFN2*). According to the previous studies, autosomal dominant inherited CMTs are more common according to autusomal recessive inherited ones[6]. Due to the prevalence of consanguineous marriages in the Turkish population and the family structure with many children, it is estimated that the autosomal recessive inherited forms of CMTs may have a higher rate, unlike the literature.

By using targeted gene panels, the rate of VUS has been increased. Comments about VUS are still a diagnostic challenge in NGS method. Different laboratories report different comments about the same variant. Hence, it is important to know the effect of VUS for an effective genetic counseling. If there is sufficient data about VUS, it can also be evaluated as benign or likely benign polymorphisms. If there is more than one VUS in a patient, this can affect the disease burden and also explain the variable expressivity at the phenotypes of the patients[16]. VUS variants need to involve a multidisciplinary medical team for phenotype-genotype correlation.

In our study, VUS were identified in 13% of the patients in *MFN2*, *SBF2*, *ARHGEF10*, *VCP*, *PMP22*, *TRPV4*, *SBF1*, SH3TC2, *REEP1*, *FIG4*, *INF2*, *PLEKH5* (2), *DYNC1H1*, *DHTKD1*, *GARS1*, and *EGR2* genes. The single variants in dominant genes associated with CMT were more common (8/14 genes). Only the *PLEKHG5* gene that had autosomal recessive inheritance had two heterozygote VUS, and the other genes had only one VUS. Three patients were co-segregated with the healthy consanguineous obligate carrier parent. Some families of the other patients were not available or could not be reached; hence, family study could not be carried out on these patients, but they are considered to be recalled. Patients were offered an annual follow-up evaluation for VUS. In different CMT-NGS studies, various results were reported for VUS according to population diversities[16-18]. Larger population-based studies could reduce the prevalence of VUS.

WES was also performed in our study, as an advanced examination in five of the male patients whose panel results were found to be normal. The mean age of these patients was 18.6 years. Four patients had homozygote pathogenic/likely pathogenic variants at different genes (*VAMP1*, *MRM2*, *PLA2G6*, and *MME*), which had all autosomal recessive manner. A novel mutation at splice site of *MME* gene was evaluated as likely pathogenic. All of these five patients had neuropathic changes at their EMGs. WES captures and sequences only 1–2% of the entire genome. Over the past decade, WES has been a very

popular research tool and the main driver in the identification of new CMT-related genes. WES also allows sequencing of genes that have never been associated with CMT or other Mendelian diseases. Since neuropathy can accompany other neuromuscular diseases besides CMT, results other than CMT can also be obtained by WES. Different groups report diagnosis rates as 19–45% in people with CMT or complex neuropathy who had negative genetic tests earlier[19]. In our study, WES was performed on molecularly undefined patients with CMT. The diagnosis rate among the patients who underwent WES was 80%. We think that the increased rate is the result of appropriate patient selection and the previous negative genetic tests.

Two novel mutations at *INF2* and *EGR2* genes related to CMTDIE and CMT1D, respectively, were detected by targeted NGS gene panel. Another novel variant at *MME* gene causing CMT2T was found by WES analysis. The variants were classified as "likely pathogenic" according to ACMG criteria[11].

For our study, the diagnosis rate was 39% (25 of 64 patients) with PMP22 MLPA. The molecular diagnosis of 18 (36%) among the 50 patients was confirmed with targeted NGS panels. Four of five patients had molecular diagnosis who underwent WES analysis. The diagnostic yield was compatible with literature[20,21]. The diagnostic rate in pediatric age group (54%) was higher than adult age group (39%). Especially, pediatric patient group that targeted NGS did not diagnose were surprisingly diagnosed by WES.

Out of 64 patients who first applied to our clinic for neuropathy, 25 were diagnosed by MLPA, 18 by targeted NGS panel (out of a total of 50 patients with a normal *PMP22* MLPA result who were consulted to our laboratory for further genetic analysis), and 4 by WES. As a result, the diagnostic yield of our study was 73% (47 patients). Thus, it can be said that the algorithmic molecular approach increases the diagnosis rate in hereditary neuropathies.

CONCLUSION

Gene panels provide excellent capture of intended CMT-associated gene regions, so they minimize false negatives with uniform coverage and high reading depths. The diagnostic rate for CMT gene panels ranges between 18 and 31% in the literature, depending on the CMT cohort, demographic background, sequencing platform, and number of genes included. The most important point is to evaluate the bioinformatics analysis of the variants obtained by NGS in correlation with the clinics of the patients.

In our study, targeted NGS panel was diagnostic in nearly one-third of the patients with CMT clinics after the exclusion of *PMP22* deletion/duplication analysis. WES is an advanced technique in patients with negative targeted gene panels and *PMP22* gene duplication/deletion. The molecular etiology in CMT patients can be determined according to pre-test evaluation, deciding the inheritance type with pedigree analysis, clinical phenotype, and an algorithmic molecular approach for the genetic analysis. Early onset of the disease, consanguinity marriage, or positive family history is important for a correct genetic diagnosis. An accurate diagnosis is also important for an appropriate genetic counseling for the patients to understand the significance of genetic testing. As in our study, the presence of patients without a molecular diagnosis in all the literature suggests that new genes or mechanisms are needed to be discovered in the etiology of CMT.

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INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

AUTHORS' CONTRIBUTIONS

GGC: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **EH:** Data curation, Formal Analysis, Investigation. **BÇ:** Data curation, Formal Analysis, Methodology, Software, Writing – original draft. **ET:** Data curation, Formal Analysis, Methodology. **CNSG:** Data curation, Writing – review & editing. **ÖYK:** Funding acquisition, Resources, Visualization, Writing – original draft. **SB:** Resources, Visualization, Writing – original draft.

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